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ADA2 isoform of adenosine deaminase from pleural fluid

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Abstract Adenosine deaminase isoenzyme 2 (ADA2) was isolated from human pleural fluid for the first time. Molecular and kinetic properties were characterized. It was shown that the inhibitors of adenosine deaminase isoenzyme 1 (ADA1), adenosine, and erithro-9-(2-hydroxy-3-nonyl)adenine (EHNA) derivatives are poor inhibitors of ADA2. Comparison of the interaction of ADA2 and ADA1 with adenosine and its derivative, 1-deazaadenosine, indicates that the isoenzymes have similar active centers. The absence of ADA2 inhibition by EHNA is evidence of a difference of these active centers in a close environment. The possible role of Zn^{2+} ions and the participation of acidic amino acids Glu and Asp in adenosine deamination catalyzed by ADA2 were shown.

Keywords: Tuberculous pleural fluid; Adenosine deaminase isoenzyme; Interaction with substrates and inhibitors

1. Introduction

Adenosine deaminase (ADA, EC 3.5.4.4), a key enzyme in the purine salvage pathway, catalyzes adenosine and 2′-deoxyadenosine (2′-dAdo) deamination to inosine and 2′-deoxyinosine [1]. It is widely distributed in mammalian tissues, and plays a critical role in proliferation, maturation and function of lymphoid cells. Congenital ADA deficiency is accompanied by accumulation of both intracellular and extracellular adenosine (Ado), 2′-dAdo, and their cytotoxic metabolites, by T-cell depletion [2,3], and frequently is associated with severe combined immunodeficiency syndrome [4]. As nucleoside related drugs are now being used in clinics, the role of ADA has become more significant as a metabolic link that decreases the lifetime of chemicals and hence shortening the time of their therapeutic action.

Three molecular isoforms of human ADA are known: two ADA1 forms – a low molecular weight form (34–43 kDa), a high molecular complex with the ADA-binding protein (280–300 kDa), and ADA2 (110 kDa). ADA1 isoforms are extensively studied, but there is only limited information known about ADA2.

ADA2 differs from ADA1 by kinetic and immunochemical properties and appears to be encoded by a separate genetic locus [1,5]. Its cellular source is not known and its physiological role is poorly understood. ADA2 is responsible for a minor part of total ADA activity in most tissues [6,7], but it is the predominant isoenzyme in the serum of normal subjects [8]. The level of ADA2 activity in serum changes at some physiological states, for example, levels were shown to decrease during normal pregnancy [9]. The increase of ADA2 activity during a number of diseases (HIV-infection, AIDS, autoimmune abnormalities, and tuberculosis) implies that it is involved in their development [10–14].

Using mathematical modeling, Gakis et al. [15] showed that isoenzymes with different affinities for a substrate form a homeostatic mechanism, constructing a biochemical enzyme–substrate system capable of preserving the cell against environmentally induced changes. One example is the system of two isoenzymes, ADA1 and ADA2, that have different affinities for two cytotoxic substrates, Ado and 2′-dAdo. This system can guarantee optimal levels of these substrates in the cell in spite of their continuous supply from the environment.

The investigation of the fine mechanism of ADA isoenzymes interaction with the inhibitors is important for the design and synthesis of new inhibitors as prospective as drugs to regulate the isoenzymes at pathology. We have previously addressed this question by studying the interaction of low molecular ADA1 from different tissues with derivatives of Ado and erithro-9-(2-hydroxy-3-nonyl)adenine (EHNA) [16].

The difference in the physiological roles of ADA1 and ADA2 is thought to be based on diversities in their molecular and kinetic properties. The aim of the present study was the isolation and purification of ADA2 from human tuberculous pleural fluid, and a comparison of its molecular and kinetic properties with ADA1 and ADA2 from human blood serum [5,17].

2. Materials and methods

2.1. Chemicals and devices

The tuberculous pleural fluid was taken at the State Clinical Tuberculous Hospital of Armenia and stored at −12 °C until use.

Ado, 2′-dAdo, dipeptide Gly-Pro-pNA, BSA, ovalbumin, EHNA, DTT, phenylmethanesulphonylfluoride (PMSF), Blue Dextran, Coomassie brilliant blue G-250 were purchased from Sigma (St. Louis, MO, USA); CM-Sephadex C50 (C50) and Sephadex G200 superfine (G200) were purchased from Pharmacia Biotech (Uppsala, Sweden); chemicals for electrophoresis were from Reanal (Hungary). Derivatives of Ado, 1-deazaadenosine (1-dAdo) and 3-deazaadenosine

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Abbreviations: ADA1, adenosine deaminase isoenzyme 1; ADA2, adenosine deaminase isoenzyme 2; Ado, adenosine; 2′-dAdo, 2′-deoxyadenosine; C50, CM-Sephadex C50; EHNA, erithro-9-(2-hydroxy-nonyl)adenine; G200, Sephadex G200 superfine; 1-dAdo, 1-deazaadenosine; 3-dAdo, 3-deazaadenosine; 3-dEHNA, 3-deazaEHNA; PMSF, phenylmethanesulphonylfluoride

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(3-dAdo), and of EHNA, 3-deazaEHNA (3-dEHNA), were synthesized and provided by Prof. Gloria Cristalli (University of Camerino, Italy). Adrenodoxin and cytochrome *c* were purified in our laboratory.

Spectrophotometer measurements were carried out on Specord M-40, (Carl Zeiss, Jena, Germany); cuvettes with light path 1, 2, 5, and 10 mm were used in thermostated cuvette holder. Centrifugation was provided on centrifuges K-23 and K-24 (Heinz Janetzki KG, Leipzig, Germany). Microconcentration tubes were from 'Centricon' (Amicon, Danvers, USA).

2.2. Enzyme purification

Pleural fluid was collected and immediately frozen. For enzyme purification 150 ml of pleural fluid was thawed, homogenized in a Porter glass homogenizer, and dialyzed for 15 h against 20 volumes of 20 mM potassium-phosphate buffer, pH 6.0 (buffer A), containing 1 mM PMSF, 1 mM EDTA and 20 μM $ZnSO_4$ (buffer B). The precipitate was removed by centrifugation at 10 000 × *g* 10 min. The supernatant was diluted 3-fold using buffer B without PMSF and EDTA (buffer C) and applied to the C50 column (2.2 × 10 cm) that had been equilibrated with buffer C. The column was sequentially washed with buffer C, containing KCl at concentrations of 0.05, 0.1 and 0.2 M. Afterwards, the fractions containing ADA2 activity were eluted using linear gradient of KCl concentrations from 0.2 to 0.4 M in buffer C (total volume 300 ml). These fractions were diluted 10-fold with buffer C without KCl, concentrated on a small column C50, and applied to a gel-filtration column of G200 (1.5 × 90 cm). The central fractions possessing ADA2 activity were concentrated by centrifugation in a microconcentrator at 3000 × *g*, 4 °C. The obtained preparation was analyzed on homogeneity by PAAG electrophoresis.

2.3. Enzyme assay

ADA activity was assayed by evaluation of liberated ammonia using the colorimetric method of Chaney and Marbach [18] described in our previous study [19]. Briefly, each of the two identical incubation mixtures contained in 350 μl: 40 mM phosphate buffer, pH 6.0, the appropriate amount of a sample, and 40 μM EHNA in one of these parallel mixtures. After preincubation at 25 °C for 20 min, the enzymatic deamination was initiated by addition of 150 μl of Ado stock solution (20 mM in distilled water) and incubated for 40 min at 37 °C. The reaction was stopped by addition of 1 ml of both the phenol–nitroprusside and hypochlorite reagents for ammonia determination. The amount of ammonia was calculated based on the absorption at 625 nm, using ammonium sulfate as a standard. The resulting value for the assay mixture without EHNA was defined as 'total ADA activity'. The value for the EHNA containing parallel mixture was defined as the activity of ADA2. The difference between these two values was defined as activity of ADA1.

2.4. Determination of pH-optimum

The dependence on pH at the range of 3.5–8.5 of ADA2 activity in the reaction of adenosine deamination was studied. 40 mM succinate–HCl buffers (pH 3.5–5.5) and 40 mM K–Na phosphate buffers (pH 5.5–8.5) were used. The assay mixture was preincubated at the appropriate pH before beginning the enzymatic reaction by addition of substrate (see above).

2.5. Determination of inhibition constant

The value of the inhibition constant, K_i, was determined by using the Dixon graphical approach [20]: reciprocal activity was plotted as a function of inhibitor concentration at two different concentrations of substrate (usually, 3 and 6 mM). The K_i for competitive inhibitor was determined as the negative value of the abscissa of interception of these two linear plots.

2.6. Other methods

Electrophoresis in 7.5% PAAG at room temperature was conducted in accordance with the standard procedure of Reisfeld for alkaline proteins [21]. 20–50 μg of protein sample was applied to each row. After electrophoresis (55 mA, 3 h), the gels were fixed in 10% TCA and visualized with Coomassie Brilliant Blue G 250.

Protein was determined by the method of Bradford [22] with BSA as a standard.

The kinetic and statistical analyses were performed with GraFit [23] and InStat (version 3 for Windows) software. All parameters were estimated based on at least three independent purification procedures.

3. Results and discussion

3.1. Purification of ADA2 from pleural fluid

We have previously shown that the ADA2 isoform is responsible for up to 80% of the total ADA activity in pleural effusions of tuberculous patients [24,25]. Therefore, we used pleural fluid as the source of this isoenzyme.

As the first step of ADA2 purification, cation-exchange chromatography on C50 column was used. In the fractions that passed through the column during application of dialyzed pleural fluid and washing with buffer C, the ADA activity was inhibited by EHNA, a well known selective inhibitor of ADA1. These fractions were collected as isoenzyme ADA1. Contaminating protein without ADA activity was removed at washing the column with buffer C, containing 0.05, 0.1, and 0.2 M KCl. The elution diagram of the KCl concentration gradient between 0.2 and 0.4 M in buffer C is shown in Fig. 1. The deaminating activity in these fractions was not inhibited by EHNA and we collected it as the ADA2 isoenzyme. As this step completely separated the two ADA isoenzymes, no further testing of the protein preparation activity with EHNA was required.

The central active fractions were collected, concentrated on a second small column of C50, and applied to a gel-filtration column of G200. PAAG electrophoresis of central fractions

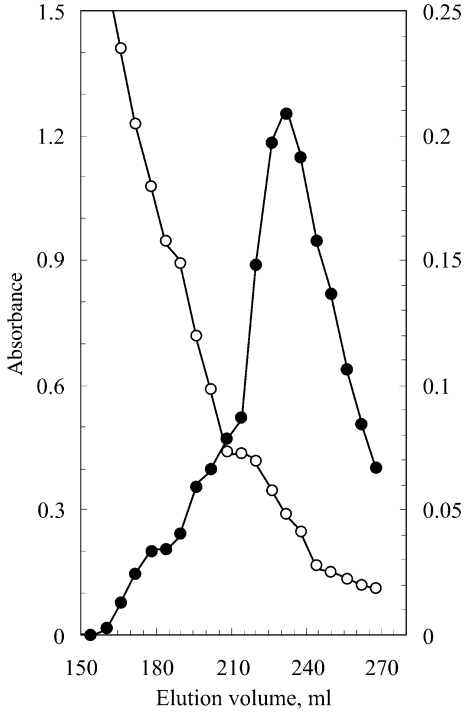

Fig. 1. ADA2 elution diagram from CM Sephadex C50 column at application of linear gradient of KCl concentrations from 0.2 to 0.4 M in buffer C. (-○-) – protein absorbance at 280 nm; (-●-) – ADA activity determined in the presence of EHNA (absorbance at 625 nm).

after this procedure showed the homogeneity of the preparation (Fig. 2, Table 1).

In contrast to ADA1, ADA2 appeared more labile. It drastically lost enzyme activity upon freezing. Taken together with the low level of ADA2 in most tissues, this instability also presents difficulties in both the purification and study of the catalytic properties. However, it is noteworthy that our experiments proved that the presence of 20 μM zinc ions in buffers improved the stability of ADA2 during purification. This observation assumes the involvement of Zn^{2+} ions in ADA2 active center structure, similar to ADA1 [26] and AMP deaminase [27].

3.2. Molecular mass

The gel-filtration on a G200 column was used for evaluation of molecular mass of ADA2. The column was calibrated using BSA (M.m. of dimer –134 kDa and of monomer – 67 kDa), ovalbumin (44 kDa), adrenodoxin (14 kDa) and cytochrome *c* (12 kDa) as markers. The void volume (V_0) of column was evaluated with blue dextran (M.m. 10^6 Da) and the dependence of ratio of the markers' elution volumes to V_0 versus the logarithm of molecular mass was plotted. From the linear graph of this dependence (not shown), the M. m. of ADA2 was estimated as 107 kDa. This value corresponds to the known value for the enzyme from human blood serum, 110 kDa [5].

3.3. Kinetic parameters

We compared the enzymatic catalysis of deamination of two substrates, Ado and 2′-dAdo.Both of these deamination reactions fit theMichaelis–Menten rule. The double reciprocal linear plots of activity vs substrate concentration (Lineweaver and Burk plots) allowed evaluation of the Michaelis parameters, k_{cat} and K_m (Table 2). The Michaelis coefficient of ADA2 for Ado, K_m = 1.48 mM, was by 1.5 orders of magnitude higher than K_m of ADA1 (0.1 mM [28]), and is close to the value for ADA2 from human serum [17]. K_m values for Ado and 2′-dAdo are practically the same. However, the higher value of catalytic efficiency, k_{cat}/K_m, for Ado deamination in comparison with that for 2′-dAdo (last column in Table 2) makes it a more preferable substrate for ADA2.

Fig. 2. PAAG electrophoresis of purified ADA2 from human pleural fluid.

3.4. pH-optimum

In Fig. 3, the dependence on pH of ADA2 activity in the deamination of Ado is shown. It demonstrates the broad pH-optimum of isoenzyme activity at pH ranges between 5.5 and 7.0. The bell-shaped pH-profile drastically decreases at higher pH, remaining gently sloping at the acidic region. It is worth noting that the pH-dependence of low molecular weight ADA1, having a broad range of pH-optimum between 7.0 and 8.5, shows a more drastic decrease to the acidic pH [29].

The obtained pH-profile was analyzed using GraFit software, and two pK_a values on the gently sloping acidic side, 4.1 and 4.5, and one on the basic side, 7.8, were evaluated. The pK_a values in the acidic side allow us to suggest that Asp and Glu with pK_a 3.9 and 4.3, respectively, participate in adenosine deamination catalyzed by ADA2. The same had been shown for ADA1 (Glu217, Asp 295) by crystallographic investigations [30]. Moreover, the lower value of pK_a for ADA2 compared with the value for ADA1 (5.6 [29]) led us to conclude that the role of these amino acids in ADA2 catalyzed reaction is more substantial than in the case of ADA1.

3.5. Interaction with the known inhibitors of ADA1

In this work, we tested the inhibition of ADA2 by two substrate analogs, 1-dAdo and 3-dAdo, and by EHNA and its derivative, 3-dEHNA.

The K_i value of 1-dAdo for ADA2 (80 μM, Table 2) was 1.5 orders of magnitude higher than for ADA1 (2–5 μM, [16]). The K_m values for ADA2 and ADA1 also differed by 1.5 orders of magnitude (1.5 mM, Table 2, and 0.1 mM [28], respectively). We consider the similarity of 1.5 orders of magnitude between the K_m values of adenosine and the K_i values of 1-dAdo for the two isoenzymes to be indirect evidence of similarity in the structure of the active centers of ADA1 and ADA2. A poor inhibitor of ADA1, 3-dAdo ($K_i = 1 \times 10^{-3}$ M), did not inhibit ADA2 in our experimental conditions, confirming our suggestion.

The stronger inhibitors of ADA1 EHNA ($K_i = 2-5 \times 10^{-8}$ M) and 3-dEHNA ($K_i = 1-1.2 \times 10^{-7}$ M) did not inhibit ADA2. This provides evidence of the essential difference in binding sites of EHNA in two ADA isoenzymes: the hydrophobic site on ADA1 that is required for complex formation

Table 1
Stages of ADA2 purification

Purification step	Total volume (ml)	Total protein (mg)	Activity of ADA2		Extent of purification	Yield (%)
			Total (nmol/min)	Specific (nmol/min/mg)		
Pleural exudates	150	6800	1100	0.16		
Dialysis	160	2880	1100	0.38	2.4	100
C50	16	0.8	169	203	1256	15
G-200	9.7	0.25	146	586	3615	13

Table 2
Steady-state kinetic parameters for deamination of substrates by ADA2 and for its interaction with inhibitors

Substrate (inhibitor)	K_m (mM)	K_i (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}\ s^{-1}$)
Adenosine	1.48 ± 0.12[a]	–	13.3 ± 1.1	8.98
2′-dAdo	1.55 ± 0.13	–	3.3 ± 0.21	2.15
1-dAdo	–	80 ± 10	–	
3-dAdo		Non-inhibited		
EHNA		Non-inhibited		
3-dEHNA		Non-inhibited		

[a]The parameters are presented as means of at least three independant determinations ±S.E.

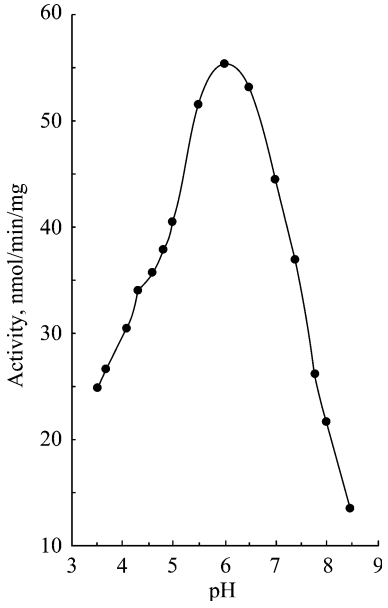

Fig. 3. The dependence of ADA2 activity on pH.

with the aliphatic chain of EHNA is probably absent in ADA2.

4. Conclusion

For the first time, ADA2 from human pleural fluid has been isolated and characterized. The molecular and kinetic properties were studied, and their identity to main characteristics of ADA2 from human plasma is demonstrated. The differences in interaction of ADA1 and ADA2 with Ado and its derivative 1-dAdo suggest the similarity of structure of their active centers. The failure of a strong ADA1 inhibitor, EHNA, to inhibit ADA2 suggests the absence of hydrophobic site in ADA2 preventing this compound binding. The probability of Zn^{2+} ions involvement in ADA2 active center structure and the importance of amino acids Glu and Asp in ADA2 catalyzed reaction are suggested.

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